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PRINCIPAL INVESTIGATOR: Mira O. Jung, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057-1411

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Failure of conventional treatment of prostate cancer with radiotherapy may be due to intrinsic resistance of the tumor cells. One of mechanisms underlying intrinsic radiation sensitivity is linked to the state of chromatin architecture. The long-term goal of this proposal is to develop a novel therapeutic strategy by enhancing radiosensitivity of prostate cancer cells by testing the hypothesis that an increase of cellular radiation sensitivity may be achieved by exposure of cells to specific HDAC inhibitors. During the second year of the research funding period, the major accomplishment and significance of the research include:(1) siRNA HDAC isoform transfection and determination of efficacy of siRNA HDAC isoforms on cellular radiation sensitivity. (2) Determination of gene expression profiling regulated by HDAC inhibitor, TSA, and by HDAC isoforms.

15. SUBJECT TERMS
Radiation sensitivity, histone deacetylase, cytotoxicity

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INTRODUCTION

Our long-term goal of this research is to develop a novel therapeutic strategy by enhancing the radiosensitivity of prostate cancer cells using low concentrations of radiosensitizer and radiation, thereby reducing radiation damage to normal tissue. The major hypothesis to be tested is **that an increase of cellular radiation sensitivity may be achieved by exposure of cells to certain HDAC inhibitors, leading to a potential clinical translation in the combined modality treatment of prostate cancer.**

BODY

During the second year of the research funding, as outlined in Task II of the statement of work (S.O.W.), we proposed to identify and validate potential target HDAC isoforms for actions of confined HDAC inhibitors. The experimental plans include: (a) identifying specific isoforms targeted by HDAC inhibitors to enhance radiosensitization of cells. (b) Limited target specificity of HDACs in response to various HDAC inhibitors will also be evaluated by analyses of gene expression profiles.

The major accomplishments associated with Task II outlined in the S.O.W. include:(1) siRNA HDAC isoform transfection and determination of efficacy of siRNA HDAC isoforms on cellular radiation sensitivity. (2) Determination of gene expression profiling regulated by HDAC inhibitor, TSA, and by HDAC isoforms.

Tranfection of siRNA HDAC isoforms in PC3 cells:

To determine the role of specific HDAC isoforms in cellular radiosensitivity, PC3 cells were transfected with siRNA duplexes (Dharmacon). The inhibition of the target isoform expression was confirmed by performing immunoblotting and confocal microscopic analyses. The protein expression levels of a number of HDAC isoforms were markedly reduced. As shown in Figure 1, the class I HDAC 1, 2, 3, 8 and class II HDAC7 proteins were successfully depleted by siRNA. We also acknowledged that this method does not apply to some of HDAC isoforms whose expressions are very low in this cell line, such as HDAC4 and HDAC8. Efficacy of siHDAC1 or siHDAC3 was further confirmed by immunocytochemistry and confocal analyses (shown in Appendices).

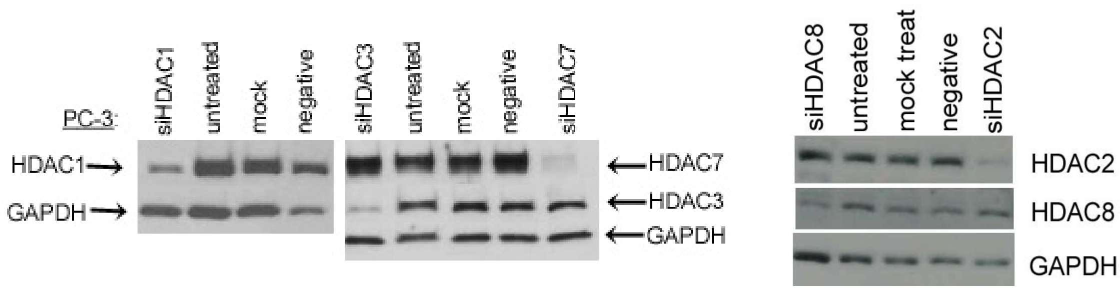


Figure 1. Effects of siRNA HDAC isoforms on the endogenous HDAC protein expressions. siRNA HDAC oligomers were transfected for 72 hours in PC3 cells. The protein expression levels of corresponding proteins were determined by Western analyses. Cell extracts prepared from mock and negative siRNA oligomers transfected and untreated cells were used as controls. An antibody to GAPDH was used as a loading control.

Effects of candidate HDACIs and siRNA HDAC isoforms on radiation clonogenic survivals:

Previously, HDAC inhibitory activities of biarylalanine analogues of candidate HDAC inhibitors (SW55 and its derivatives, ST groups) were assessed. These compounds exhibited the IC_{50} HDAC inhibition values at nano-molar concentrations. To test the ability of HDAC inhibitors to sensitize cells to radiation, effects of the compounds on cellular radiosensitivity were evaluated by performing clonogenic survival assays. Based on previous data, we focused on efficacy of SW55 and ST12, which confer the most potent inhibitory activity among derivatives tested. Radiation clonogenic survival assays were performed following treatment of cells with the IC_{50} values of these HDAC inhibitors. Surviving colonies were scored and radiation sensitivity was determined by using the single hit multiple-target and the linear quadratic models. In Table 1, radiosensitivity of PC3 cells with HDAC inhibitors, SW55 and ST12, was compared to parental cells (D_0=1.5 Gy). The values of D_0 were significantly decreased in the combined treatment (showing radiation sensitization): 1.28 Gy by SW55 and 1.12 Gy by ST12. In addition, the efficiency of survival colony formation was only 10% by 1 Gy of gamma radiation in combination with SW or ST12 treatment while radiation alone confers 55% survivals, supporting our strategy of using the HDAC inhibitor as a radiosensitizer.

We further examined transfected cells with siRNA targeting specific HDAC isoforms to address which depletion of specific isoforms causes alterations in the biological phenotypes associated cellular radiosensitivity. As shown in Table 1, cells transfected with siRNA HDAC1 as well as mock treated cells confer no change in radiation sensitivity (Do=1.52 Gy) while cells with siRNA HDAC3 and HDAC7 exhibit moderately reduced sensitivities (Do=1.43 for siHDAC3 and Do=1.37 Gy for siHDAC7). The sensitization effect of siHDAC7 was similar to that by SAHA.

Taken together, these results suggest that the candidate HDAC inhibitors are effectively sensitize PC3 cells to radiation. It is of particular interest that there is a disparity in radiation sensitizing effectiveness between these compounds. Therefore, we will mainly focus on characterization of ST12 in further studies.

Table 1. Effects of HDAC inhibitors in PC cells (D_0=1.49 Gy)

Compound	50% HDAC inhibition activity	Cytotoxicity (IC_{50})	Radiation Sensitivity (Gy)
TSA	10 nM	0.3 μM	1.10 Gy
SAHA	170 nM	1 μM	1.32 Gy
SW55	290 nM	1 μM	1.28 Gy
ST12	30 nM	1.8 μM	**1.12 Gy**
SiHDAC1	-	-	1.52 Gy
SiHDAC3	-	-	1.43 Gy
SiHDAC7	-	-	1.37 Gy
mock			1.54 Gy

Identification of specific HDAC isoforms targeted by HDAC inhibitor, ST12:

To identify specific HDAC isoforms targeted by candidate HDACIs, we focused on class I HDAC isoforms. *In vitro* HDAC activity assays were performed using immunoprecipitates (HDAC1, HDAC2, HDAC3) or recombinant protein (HDAC8) of class I HDAC isoforms. The data show that SW55 inhibited ~70-80% of HDAC1, HDAC2, HDAC3 activities and 20% of HDAC8 (TSA and SAHA confer 10% and no inhibitory activity, respectively). ST12 inhibited ~60-70% of HDAC1, HDAC2, HDAC3 and ~40% of HDAC8. These data suggest that the compounds, SW55 and ST12, non-specifically target class I HDAC1, HDAC2, and HDAC3. Such results are possibly due to the nature of immunoprecipitates, which brought down a complex form of these isoforms together. However, ST12 was more potent against recombinant HDAC8 while TSA and SAHA show little inhibitory activity. Therefore, these results need to be further confirmed in cultured cells.

Effects of HDACs on Gene expression profiling:

To explore the biological pathways in which the target is involved, we analyzed the gene expression profiles following exposure to HDAC inhibitors. Genes of interest will be confirmed for differential expression using Real-Time PCR and immunoblotting.

PC3 cells were initially analyzed by utilizing Affymetrix microarrays (U133A) after treatment with the HDAC inhibitor. cRNA fragments will be prepared as suggested by the manufacturer (Affymetrix). To obtain statistic significance, four replicates per experimental data point from independent experiments were run for each of samples. The computational programs for the data analyses include the Affymetrix Data Mining Tool, the GeneSpring program and MatLab, which are frequently used in our lab.

Following normalization and filtering of each chip and gene, the gene expression levels were examined utilizing parametric Welch's parametric t-test ($p \leq 0.05$) to compare samples. The fold change ratio was then computed relative to the cell lines initial expression level. Functional clustering was applied to the pool of genes that changes significantly at least once during treatment in samples. Venn diagramming was utilized to cross reference the genes that differed significantly at indicated intervals in order to select genes that change significantly after treatment. Interestingly, a number of genes changed only in cells transfected with siRNA HDAC7 were significantly higher (**1474** genes at 67% up-regulated and 33% down-regulated) than those by siRNA HDAC1 (**416** genes at 61% up-regulated and 39% down-regulated) and HDAC3 (**420** genes at 60% up-regulated and 40% down-regulated). The data suggest that these HDAC isoforms function not only down regulating gene expressions, but are also involved in gene activation. Currently, we are confirming the data by measuring the expression levels of a number of genes, which are significantly up or down-regulated, using Real Time (RT)-PCR and biochemical methods.

KEY RESEARCH ACCOMPLISHMENTS:

1. Determined the radiosensitizing effect of candidate HDAC inhibitors and HDAC isoforms.
2. Identified a potential role of HDAC7 in intrinsic cellular radiation sensitivity.
3. Identified specific HDAC isoforms targeted by the candidate HDAC inhibitors.
4. Identified gene expression profiling affected by HDAC1, HDAC3, and HDAC7.
5. Identified a lead compound for animal testing proposed in Task III.

REPORTABLE OUTCOMES:

1. Manuscripts in preparation.

CONCLUSIONS

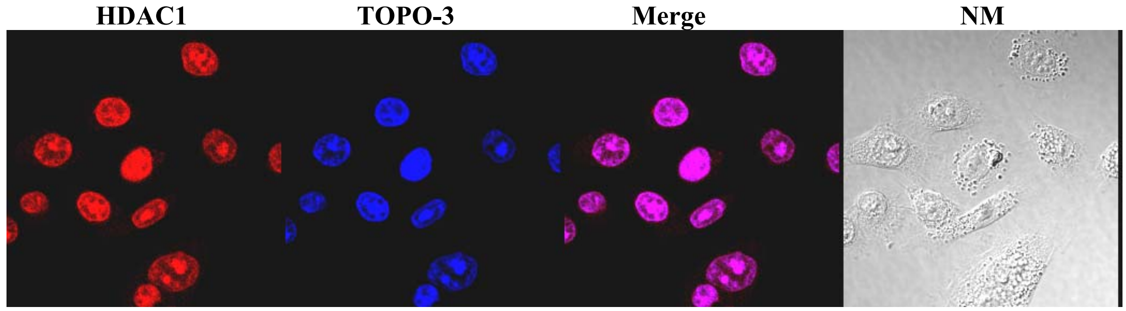
During the second year of the research funding, we examined radiosensitizing property of the candidate HDAC inhibitors in PC cells. As shown in Table 1, SW55 and ST12 significantly increased radiosensitization of cells. Cells transfected with siRNA HDAC7 also exhibit radiosensitizing effect albeit at the moderate level. Although no propounding efficacy was observed, the radiosensitizing effect by siRNA HDAC7 is significant in that such moderate effect is due to the heterogeneous cell population, some of which are escaped from siRNA transfection. Furthermore, the candidate HDAC inhibitors, SW55 and ST12, target class I HDAC isoforms. In the remaining funding period, we will focus on charactering ST12 and validating a number of genes regulated by HDAC isoforms obtained from gene expression profiling analyses. We will also test ST12 as a lead compound in the animal model proposed in Task III.

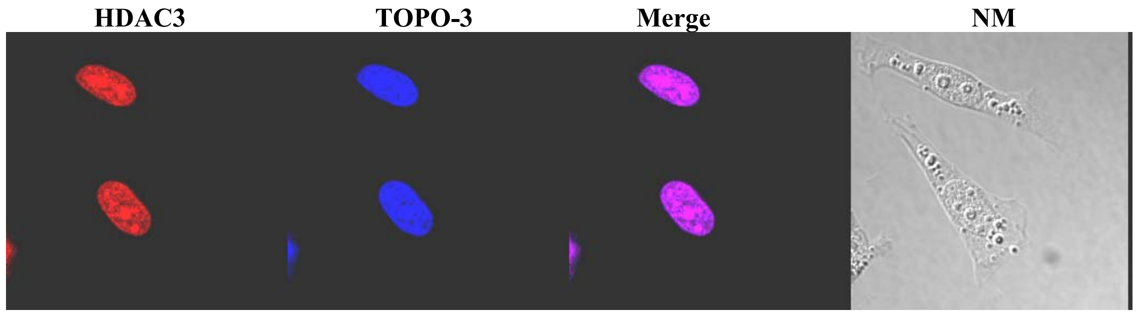
REFERENCES:

APPENDICES:

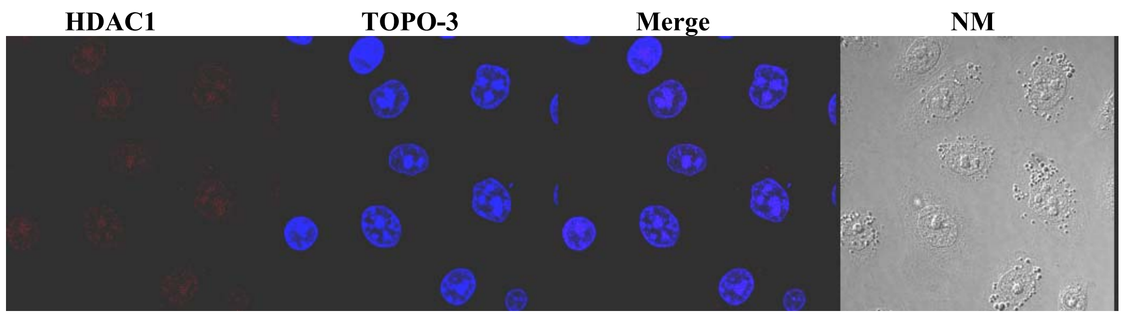
1. Immunocytochemistry and confocal data:

PC3 CELLS:





PC3/SIHDAC1:



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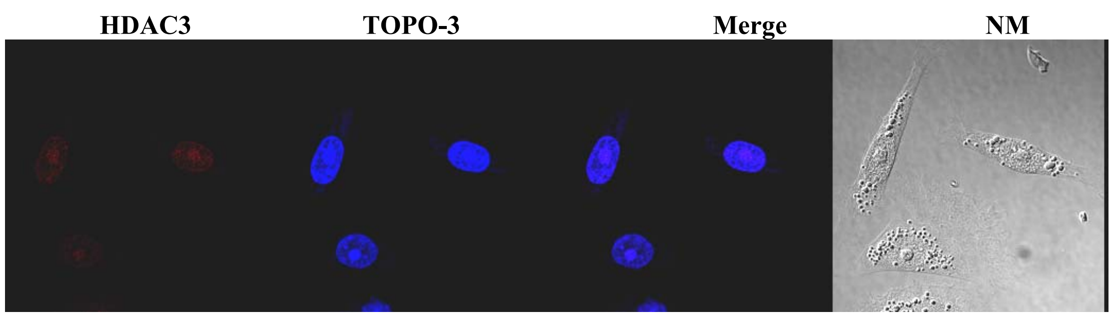

2. Gene expression data:

PC cells transfected treated with TSA for 24 hours or with siRNA HDAC1, HDAC3, or HDAC7 were analyzed for their gene expression profiling. Tables show % of up- or down-regulated genes and a number of genes in various functional groups.

A. Significantly regulated genes following exposure of PC3 cells to TSA.

Control = untreated PC3 cells		Up-Reg	Down-Reg
	# genes	# genes (%)	# genes (%)
TSA vs Control: (p<= 0.05 AND |Fold| >= 2.0 AND Flag Filter) **AND** . **TSA vs DMSO**: (p<= 0.05 AND |Fold| >= 2.0 AND Flag Filter)	**413**	155 **(38%)**	258 **(62%)**

B. Significantly regulated genes in various functional groups.

	TSA
apoptosis regulator activity (GO:0016329)	10
catalytic activity (enzymes)	102
cell adhesion molecule activity (GO:0005194)	8
cell cycle (GO:0007049)	17
cell death (GO:0008219)	10
cell differentiation (GO:0030154)	21
cell growth (GO:0016049)	38
cell proliferation (GO:0008283)	10
chromatin binding (GO:0003682)	0
cytoskeleton (GO:0005856)	11
defense immunity protein activity (GO:0003793)	7
DNA Binding	36
plasma membrane (GO:0005886)	42
regulation of gene expression, epigenetic (GO:0040029)	41
signal transduction (GO:0007165)	28
structural molecule activity (GO:0005198)	16
transcription factor activity (GO:0003700)	36
transporter activity	41
mitochondrion (GO:0005739)	16
metabolism	100
extracellular (GO:0005576)	13

C. Significantly regulated genes in PC3 cells transfected with siRNA targeting HDAC1, HDAC3, or HDAC7.

		Up-Reg	Down-Reg
	# genes	# genes (%)	# genes (%)
(siHD1 vs Control) only	416	252(61%)	164(39%)
(siHD3 vs Control) only	420	252(60%)	168(40%)
(siHD7 vs Control) only	1474	989(67%)	485(33%)
(siHD1 vs Control) AND (siHD3 vs Control)	126	84(67%)	37(29%)
(siHD1 vs Control) AND (siHD7 vs Control)	391	194(50%)	187(48%)
(siHD3 vs Control) AND (siHD7 vs Control)	202	117(58%)	70(35%)
(siHD1 vs Control) AND (siHD3 vs Control) AND (siHD7 vs Control)	254	182(72%)	62(24%)

D. Significantly regulated genes in various functional groups.

	siHDAC1 ONLY	siHDAC3 ONLY	siHDAC7 ONLY	siHDAC1 & siHDAC3	siHDAC1 & siHDAC7	siHDAC3 & siHDAC7	siHDAC1 & siHDAC3 & siHDAC7
apoptosis regulator activity (GO:0016329)	3	13	12	1	0	18	12
catalytic activity (enzymes)	119	95	432	28	116	58	61
cell adhesion molecule activity (GO:0005194)	15	19	44	3	14	15	8
cell cycle (GO:0007049)	26	25	82	14	28	13	24
cell death (GO:0008219)	15	19	48	7	12	21	8
cell differentiation (GO:0030154)	26	26	92	11	24	18	11
cell growth (GO:0016049)	50	51	168	14	52	25	31
cell proliferation (GO:0008283)	12	23	68	3	23	17	9
chromatin binding (GO:0003682)	1	11	10	3	2	2	6
cytoskeleton (GO:0005856)	13	16	55	4	25	7	8
defense immunity protein activity (GO:0003793)	25	9	45	2	10	17	13
DNA Binding	44	71	210	21	58	24	40
extracellular (GO:0005576)	16	27	69	8	22	26	9
metabolism	127	115	461	36	105	63	62
mitochondrion (GO:0005739)	12	13	64	2	13	5	8
plasma membrane (GO:0005886)	51	83	284	15	82	35	31
regulation of gene expression, epigenetic (GO:0040029)	63	91	218	22	52	47	48
signal transduction (GO:0007165)	50	64	166	12	68	25	15
structural molecule activity (GO:0005198)	11	24	63	6	19	13	13
transcription factor activity (GO:0003700)	44	60	204	19	57	22	36
transporter activity	44	54	144	16	46	28	14